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Measure of O⁶-Alkylguanine-DNA Alkyltransferase Activity in Normal Human Epidermal Keratinocytes in Culture and Effects of *Bis*-(2-chloroethyl) sulfide on the Activity

Ellen M. Kurt
Vincent M. O'Donnell
Charise C. Davenport
Agnes Gaweska

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U.S. Army Medical Research
Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010-5400

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13. ABSTRACT (<i>Maximum 200 words</i>) O ⁶ -Alkylguanine-DNA alkytransferase (AGT) is a DNA repair protein that removes alkyl adducts from O ⁶ -alkylguanine in DNA. AGT may be important in DNA repair following injury induced by <i>bis</i> -(2-chloroethyl)sulfide (sulfur mustard, HD), since O ⁶ -alkylguanine is one of the HD alkylation products. One model for HD-induced injury uses normal human epidermal keratinocytes (NHEK) in culture. In this study we measured the levels of AGT in NHEK grown to either 60- 80% or to 100% confluence and then studied the effects of a one-hour exposure to 50, 100, and 300 μM HD on AGT activity. Mean AGT activity in NHEK grown to 60-80% and 100% confluence was 470 ± 404 and 518 ± 737 fmol/mg protein, respectively. In general, AGT activity appeared to increase after exposure to 100 μM HD and decrease with increased confluence and after exposure to 50 and 300 μM HD. However, a two-way analysis of variance for cell confluence and HD concentration showed no significant differences between cell confluenices or the different HD concentrations.			
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INTRODUCTION

Sulfur mustard (HD) is a potent alkylating agent that has a long history of use as a chemical warfare agent. Despite decades of medical research, the mechanism by which HD induces vesication is not known, and no effective antidotes are currently available. The fact that DNA is a major cellular target for HD alkylation has been well documented (Fox and Scott, 1980; Rozmiarek et al., 1973). HD alkylates DNA to form ethylthioethyl adducts to include monofunctional adducts at the O⁶ position of guanine (Papirmeister et al., 1991).

O⁶-Alkylguanine-DNA alkyltransferase (AGT) is a repair protein that acts by transferring the alkyl group from O⁶-alkylguanine to a cysteine residue in its center. The reaction is stoichiometric: each molecule of AGT removes one alkyl group, which becomes covalently bound to the AGT cysteine residue (Brent, 1985; Lindahl et al., 1988; Yarosh, 1985; Pegg and Dolan 1987). The alkylated transferase molecule is then catabolized. The cell's ability to perform this type of repair depends strictly on the level of AGT (Yarosh, 1985; Pegg and Dolan, 1987). The level of AGT varies greatly among species, and different tissues contain different levels of alkyltransferase activity (Gerson et al., 1986).

Previous studies have established that normal human epidermal keratinocytes (NHEK) in culture can serve as useful models for biochemical investigations into the toxicity of HD (Smith et al., 1990). Here we report the AGT activity in cultured NHEK grown to either 60-80% or to 100% confluence, and the effects of a one-hour exposure to 50, 100, and 300 μ M HD on the AGT activity.

METHODS

Sulfur mustard. Bis-(2-chloroethyl)sulfide (5 μ l in 10 ml of Keratinocyte Growth Medium [KGM]) was obtained from the U.S. Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD).

Cell culture. Primary cultures of adult NHEK (strain 4075) were obtained from Clonetics Corporation (San Diego, CA). Tertiary cultures of NHEK were passed into 150 cm² tissue culture flasks and grown until either 60-80% or 100% confluence. At this time 50, 100, or 300 μ M HD was added to half the flasks, while the other half of the flasks served as the controls. Seven samples were exposed to 50 μ M HD, seven samples to 100 μ M HD, and seven samples to 300 μ M HD. The flasks were left at room temperature for one hour and then harvested by trypsinization according to the procedure described by Boyce and Ham (1985). The harvested NHEK were collected in a conical tube and placed in a styrofoam box containing dry ice until AGT analysis. The cell counts, determined using trypan blue staining, ranged from 2.0x10⁷ to 4.6x10⁷ cells/culture/sample.

Assay of AGT activity. The activity of AGT in NHEK was measured in the laboratory of Dr. Eileen Dolan at the Division of Hematology-Oncology, the University of Chicago Medical Center (Chicago, Illinois) as previously described (Dolan et al., 1990). Briefly, the NHEK cells were incubated with [³H]methylated DNA, which was prepared by allowing [³H]N-methylnitrosourea to react with calf thymus DNA. The DNA was precipitated then acid hydrolyzed, and the modified bases were separated by reverse-phase HPLC. Protein was determined by the method of Bradford (1976), and the results are expressed as fmol of O⁶-methylguanine released from the DNA substrate per mg protein. Each sample was run in

duplicate. The results are reported as the mean \pm the standard deviation. The AGT activity in NHEK exposed for one hour to 50, 100, or 300 μM HD is reported as percent change of control where % change = [(control-HD)/control] \times 100.

RESULTS

Table I shows the mean AGT activity measured for the cultured NHEK. Mean AGT activity measured in 12 samples of NHEK grown to 60-80% confluence was 470 ± 404 fmol/mg protein. Mean AGT activity measured in 6 samples of NHEK grown to 100% confluence was 518 ± 737 fmol/mg protein. A two-way analysis of variance showed no significant differences between confluence levels. Because there were no significant differences, all AGT activities were grouped together and the mean calculated. The mean AGT activity for the 18 samples was 485 ± 515 fmol/mg protein, which is not significantly different from zero.

Table II shows the percent changes of the control of AGT activity in NHEK, at 60-100% confluence, for the HD-exposed samples. The percent changes of the control of the AGT activity for the 50, 100, and 300 μM HD exposed NHEK were $-18 \pm 167\%$, $+52 \pm 98.1\%$, and $-72 \pm 86.9\%$, respectively. A two-way analysis of variance showed no significant differences among the HD exposure concentrations (as compared with the controls). Because there were no significant differences among HD exposure concentrations, the percent changes for all three different concentrations were grouped together and averaged. The mean percent change following exposure of the NHEK to HD is $10.06 \pm 88.9\%$, which is not significantly different from zero.

DISCUSSION

This is the first report of AGT activity in cultured NHEK as well as in NHEK exposed to HD. The AGT activity in 60-80% confluent control NHEK (470 ± 404 fmol/mg protein) is higher than that reported for other human tissues, such as brain (67 ± 40 fmol/mg protein), lung (86 fmol/mg protein), myeloid precursors (126 ± 59 fmol/mg protein), colon (138 ± 78 fmol/mg protein), and small intestine (242 ± 168 fmol/mg protein). It is, however, comparable to that reported for T-lymphocytes (359 ± 72 fmol/mg protein) and liver (485 ± 126 fmol/mg protein) (Gerson et al., 1986).

In general, AGT activity decreased with an increase in confluence. The trends for change in AGT activity in NHEK following a one-hour HD exposure were for an increase in activity after 100 μM HD and a decrease in activity after 50 and 300 μM HD. None of these trends was supported by statistical analyses, which showed no significant differences in AGT activities between NHEK of 60-80% and 100% confluence and no significant differences in AGT activity in unexposed NHEK versus NHEK exposed to 50, 100, or 300 μM HD. While the statistical analyses show that AGT activity is not a function of cell confluence and that a one-hour exposure to 50, 100, and 300 μM HD does not affect AGT activity in NHEK in culture, the general trends do show some correlation indicating that a greater sample population may provide statistical support for the observed trends.

Table 1. Mean O⁶-Alkylguanine-DNA Alkyltransferase (AGT) Activity
in Normal Human Epidermal Keratinocytes in Culture

Number of NHEK Samples	% Confluency	AGT Activity (fmol/mg protein)
12	60 - 80	470 ± 404
6	100	518 ± 737
18 ^a	60 - 100	485 ± 515

^aThe mean AGT activity for the 18 NHEK samples.

Table II. Percent Changes of Control of O⁶-Alkylguanine-DNA Alkyltransferase Activity in Normal Human Epidermal Keratinocytes, at 60-100% Confluence, Exposed to Different Sulfur Mustard (HD) Concentrations

Number of Determinations	HD Concentrations (μ M)	Percent Change ^b
7	50	-18 ± 167
7	100	+ 52 ± 98.1
7	300	-72 ± 86.9

^aPercent change = [(control - HD exposed)/control] x 100

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